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BACKGROUND OF THE INVENTION

In the past, novel biopolymer (*i.e.* DNA, RNA or polypeptide) based catalysts have been created in several different ways. The following paragraphs describe some of these selection schemes.

(1). Binding to transition state analogs

Catalytic RNA, DNA and protein (particularly antibodies) have been isolated by this approach. It has mostly been applied to the isolation of catalytic antibodies by immunization of mice with transition state analogs (TSA), but also antibodies displayed on phage as well as RNA and DNA libraries have been challenged with TSA. The idea is that a molecule (protein, RNA or DNA) that binds a given TSA is likely to bind the substrate and stabilize the geometry and/or energetics of the transition state. This may result in catalysis.

The method does not select for catalytic activity per se, but rather for *binding* to a transition state analog (TSA). However, it has been included here as it is currently one of the most used methods to isolate novel catalysts. Problems encountered with this approach include: i) Detailed mechanistic knowledge of the target reaction is required (in order to design an appropriate TSA); ii) In many cases a TSA that adequately resembles the transition state is unobtainable or unstable; iii) It is not possible to mimic the structural and electronic *dynamics* of the reaction coordinate.

Consequently, a rather limited set of reaction types have been successfully targeted by this approach. In most cases the isolated catalysts have poor turn-over numbers.

(2). Functional tagging of active catalysts

This selection scheme has been applied to protein and nucleic acids. The substrate is designed so that a reactive product is formed during the reaction (the substrate is called "suicide substrate" or "inhibitor analog"). The reactive product is likely to react with the catalyst that produced it, to form a covalent bond. As a result, active catalysts can be separated from inactive ones by way of the attached label. Catalytic antibodies displayed on phage have been isolated by this method, and

it was shown in a model system that catalytically active and inactive proteins could be s parated using this approach. The method should allow the isolation of rare catalysts.

Important limitations with this approach include: i) For many reactions it is not possible to design an appropriate suicide substrate. ii) Successful catalysts need only perform one turnover during the selective process/round, which is typically on the order of minutes. Hence, there is no selective advantage for efficient catalysts.

(3). Continuous evolution (RNA)

RNA libraries have been designed that contain both the substrate and the potentially catalytic domain in the same molecule. RNAs capable of performing the desired reaction (typically ligation) will "activate" themselves for amplification (reverse transcription followed by RNA polymerase transcription). By adequate dilutions and additions of nucleotide precursors this continuous selection can be maintained over several hours, and then analyzed.

The method has two important limitations: i) Both the substrate and the catalyst must be a nucleic acid; ii) As the catalyzed reaction and the amplification of successful enzymes is not separated, the time of the selective step is the sum of the turn-over time of the target reaction and the time of amplification of the "activated" molecules. Thus, as the amplification is on the order of seconds, there is no selective advantage for an efficient catalysts.

SUMMARY OF THE INVENTION

The problem to be solved by the present invention is to provide a method for *in vitro* selection, from a library of catalyst molecules, of a catalyst molecule of interest having a relatively more efficient specific catalytic activity of interest, as compared to the rest of the catalyst molecules within said library, and wherein said *in vitro* selection method is characterised by that it allows multiple catalytic activity turn-overs (*i.e.* substrate to product catalytic activity turn-

overs), by the catalyst molecule of interest, before it is finally collected.

The solution is based on using a sample comprising a number of individual units in said *in vitro* selection method and further wherein said selection method is characterised by the use of one or more reagent(s) which are capable of converting a product generated by a catalyst molecule of interest back into the substrate for said catalyst of interest. See figure 3 for a graphic illustration.

Further, see figure 1 for a graphic illustration of a suitable example of said sample.

Accordingly, in a first aspect the invention relates to a method for *in vitro* selection, from a library of catalyst molecules, of a catalyst molecule of interest having a relatively more efficient specific catalytic activity of interest as compared to the rest of the catalyst molecules within said library and wherein said *in vitro* selection method is characterised by that it allows multiple catalytic activity turn-overs (*i.e.* substrate to product catalytic activity turn-overs), by the catalyst molecule of interest, before it is finally collected and wherein said method comprises following steps,

(i) placing; a sample comprising a number of individual units wherein said sample comprises a library of catalyst molecules provided in the form of individual units, wherein the individual units comprise a first type individual unit having the following general structure:

C-S,

wherein C denotes a catalyst molecule and S a substrate which is capable of being catalysed into a product by at least one catalyst comprised within said library of catalyst molecules and thereby providing the possibility of obtaining a second type individual unit comprising the general structure:

C-P,

wherein C has the meaning defined above and P is the product molecule resulting from the catalytic conversion of the substrate S of the first type individual unit; and

(a) the substrate S is attached to the catalyst in a configuration that allows catalytic reaction between the catalyst and the substrate within said individual unit; and

(b) the nature of said attachment of the substrate and the catalyst provides the possibility, by means of a characteristic of the product, of isolating an entity comprising information allowing the unambiguous identification of the catalyst molecule which has been capable of catalysing the reaction substrate molecule to product molecule;

under suitable conditions where a catalyst molecule of interest performs its catalytic activity of interest and where said method is characterised by that said sample is further under conditions wherein the product generated by a catalyst of interest are in contact with one or more reagent(s) which convert it back into the substrate S;

(ii) selecting for a catalyst of interest by selecting for one or more individual unit(s) which comprise(s) the product molecule; and

(iii) isolating an entity comprising information allowing the unambiguous identification of the catalyst molecule of interest which has been capable of catalysing multiple times the reaction substrate to product, by means of a characteristic of the product; and optionally

(iv) repeating step (i) to (iii) one or more times by using the information comprised in said entity of step (iii) to generate the catalyst molecule of interest and construct an individual unit comprising said generated catalyst molecule of interest and then using this individual unit as a starting material in said repetition step.

The term "said sample is further under conditions wherein the product generated by a catalyst of interest are in contact with one or more reagent(s) which convert it back into the substrate", according to step (i) of the first aspect of the invention, denotes any reagent(s) and any combination of

reagent(s) molecules which can catalyses th reaction product (comprised within the individual unit) (P_1) to the substrate (comprised within the individual unit) (S_1) or that participates as a reactant in the reaction P_1 to S_1. For further description of this and description of preferred embodiment of said reagent(s) reference is made to the section "Guidance to a skilled person when searching for a suitable reagent" below. Further, see figure 3 for a graphic illustration.

The term "an individual unit", comprised within a sample according to first aspect as described above, denotes an individual unit comprising the general structure as specified under point (i) in the first aspect of the invention and a substrate molecule attached to a catalyst molecule as specified under point (a) and (b) in the first aspect of the invention. See figure 2 for a suitable example of such an individual unit.

The term "an individual unit comprising the general structure: a catalyst - a substrate; or a catalyst - a product" denotes that said individual unit comprises at least one molecule of each of said identities, *i.e.* at least one catalyst molecule and at least one substrate molecule or at least one product molecule. Accordingly, said individual unit may for instance comprise more than one copy of an identical catalyst molecule or may comprise several different catalyst molecules.

Further the term "-" placed between the individual identities within said individual unit denotes that there is a physical connection between said individual identities within said individual unit, *i.e.* that there is a physical connection between a catalyst - a substrate or

a catalyst - a product.

Further, "an individual unit" as described herein denotes an individual unit wherein it is possible to physically separate said individual unit from the other different individual units, within said sample, in order to be able to isolate the separate individual unit.

The term "different individual units" denotes different individual units each independently comprising different

catalyst molecules, *i.e.* an example of two different individual units may be

(1) catalyst molecule^1 - substrate; and

(2) catalyst molecule^2 - substrate;

wherein catalyst molecule^1 and catalyst molecule^2 denotes two different catalyst molecules.

The term "a sample comprising a number of different individual units" denotes a sample comprising at least two different individual units, preferably at least 100 different individual units, more preferably at least 10.000 different individual units, more preferably at least 10^6 different individual units, even more preferably at least 10^8 different individual units, and most preferably at least 10^{14} different individual units. Basically the actual number of different individual units corresponds to the actual size of the library of catalyst molecules.

The term "a sample comprising a number of individual units" and the term "a sample comprising a number of different individual units" may be used interchangeably herein.

The term "a library of catalyst molecules" denotes a library comprising at least two different catalyst molecules, preferably at least 100 different catalyst molecules, more preferably at least 10.000 different catalyst molecules, more preferably at least 10^6 different catalyst molecules, even more preferably at least 10^8 different catalyst molecules, and most preferably at least 10^{14} different catalyst molecules.

The term "a substrate capable of being catalysed into a product molecule by at least one catalyst molecule comprised within said library of catalyst molecules" basically denotes any suitable substrate molecule. Essentially said substrate molecule is chosen according to the specific catalytic activity which it is desired to select for. For instance, if the desired catalytic activity is a protease activity then a suitable substrate may be a peptide molecule and the product will then be a degraded peptide. The terms "substrate" and "substrate molecule" may be used interchangeably.

The term "product" denotes the product obtained by the catalytic reaction substrate to product by a catalyst of

interest as specifi d herein. The terms "product" and "product molecule" may be used interchangeably.

The term "catalyst" denotes any catalyst molecule with a desired catalytic activity, such as organic and inorganic molecules, proteins, enzymes, peptides, nucleic acids, biopolymers and non-biological polymers, small organic or inorganic molecules. The terms "catalyst" and "catalyst molecule" may be used interchangeably.

The term "the substrate is attached to the catalyst in a configuration that allows catalytic reaction between the catalyst and the substrate within said individual unit" denotes a direct or indirect physical connection, within each of the individual units, between substrate and catalyst. This connection should preferably maximize productive interaction of the catalyst and the substrate, within the individual unit, while minimizing the interaction of catalysts and substrates on different individual units.

The term "the nature of said attachment of the substrate and the catalyst provides the possibility, by means of a characteristic of the product, of isolating an entity comprising information allowing the unambiguous identification of the catalyst molecule which has been capable of catalysing multiple times the reaction substrate molecule to product molecule" according to point (b) of the first aspect of the invention denotes that said entity is isolated by means of one or more characteristic of the product.

An example of a suitable characteristic of the product may be that said product does not bind to a matrix and the substrate does bind to a matrix. In this case a suitable selection protocol may be that the individual units are bound to the solid support on the form a catalyst - a substrate - matrix, and released when it is on the form catalyst - a product. For a detailed description of an example of such a system reference is made to a working example herein (*vide infra*).

Another example of a suitable characteristic of the product may be that said product is binding to a receptor as illustrated in figure 2.

The term "an entity comprising information allowing the unambiguous identification of the catalyst which has been capable of catalysing multiple times the reaction substrate molecule to product molecule" according to point (b) in the first aspect of the invention, denotes either an entity wherein said information is carried in the catalyst molecule as such or an entity comprising other kind of information providing the possibility of unambiguously identifying the catalyst. Such other kind information may for instance be an entity comprising a DNA sequence encoding a peptide or a polypeptide when the catalyst molecule of interest is a peptide or a polypeptide. An illustration of this may be when the isolated entity is a filamentous phage comprising a DNA sequence encoding a polypeptide of interest attached on the surface of said phage. See *e.g.* figure 14 and below for further details.

The term "under suitable conditions where a catalyst molecule of interest performs its catalytic activity of interest" according to step (i) of the first aspect of the invention, denotes any suitable conditions where a catalyst molecule of interest performs its catalytic activity of interest.

Such suitable conditions may be alkaline pH if the purpose of the selection is to identify a catalyst of interest having activity at alkaline pH.

The term "the catalyst molecule of interest which has been capable of catalysing multiple times the reaction substrate to product" according to step (iii) of the first aspect of the invention denotes that said catalyst molecule of interest has performed the catalytic reaction substrate to product at least two times, more preferably at least times 100 times, more preferably at least 10.000 times, more preferably at least 10^6 times, and most preferably at least 10^{10} times.

The term "repeating step (i) to (iii) one or more times by using the information comprised in said entity of step (iii) to generate the catalyst molecule of interest and construct an individual unit comprising said generated catalyst molecule of interest and then using this individual unit as a starting material in said repetition step" according to point (iv) in

first aspect of the invention denotes that said repetition may be one time, more preferably 2 times, more preferably more than 5 times, even more preferably more than 10 times, and most preferably more than 25 times.

An advantage of the method for *in vitro* selection as described above is that it allows the catalyst molecules to perform multiple turn-overs of substrate to product during one selection round (*i.e.* before the catalyst molecule(s) of interest is finally collected) (see figure 4 for an illustration).

This is fundamentally different from previous in the art described selection protocols, which either involved binding to a transition state analog of the target reaction, wherein there is no turn-over of substrate (see #1 in "Background" above) or a single turn-over of substrate (#2 and 3 in "Background" above).

Accordingly, an advantage over the art my be that minor differences in activity can be distinguished; since the selective step is reiterated many times during the selection scheme and even minor differences in catalyst activity may then be differentiated.

In a final aspect the invention relates to a method for producing a catalyst molecule of interest comprising performing the method *in vitro* selection according to the invention and the further following step,

i) producing said isolated catalyst molecule of interest in a suitable quantity of interest by a suitable production method.

In a final aspect the invention relates to a method for producing a catalyst molecule of interest comprising performing the method for multiple catalytic activity turn-over *in vitro* selection according to the invention and the further following step,

(a) producing said isolated catalyst molecule of interest in a suitable quantity of interest by a suitable production method.

DRAWINGS:

Figure 1: Graphic illustration of a suitable example of a sample according to the invention comprising a library of catalyst molecules provided in the form of individual units, wherein the individual units comprise a first type individual unit having the following general structure:

C-S,

wherein C denotes a catalyst molecule and S a substrate which is capable of being catalysed into a product by at least one catalyst comprised within said library of catalyst molecules.

Figure 2: Graphic illustration of a suitable example of an individual unit wherein

(a) the substrate S is attached to the catalyst in a configuration that allows catalytic reaction between the catalyst and the substrate within said individual unit; and

(b) the nature of said attachment of the substrate and the catalyst provides the possibility, by means of a characteristic of the product, of isolating an entity comprising information allowing the unambiguous identification of the catalyst molecule which has been capable of catalysing the reaction substrate molecule to product molecule.

Figure 3: A graphic illustration of a suitable example wherein a sample of the invention is under conditions wherein the product generated by a catalyst of interest are in contact with one or more reagent(s) which actively convert it back into the substrate.

Figure 4: A graphic illustration of a suitable *in vitro* selection scheme according to the invention, wherein said *in vitro* selection scheme is characterised by that it allows multiple catalytic activity turn-overs (*i.e.* substrate to product catalytic activity turn-overs), by the catalyst molecule of interest, before it is finally collected.

Figure 5: A figure showing preferred requirements for a reagent capable of converting the product back to the substrate as described herein.

Figures 11-13: Figures supporting the description in working example 1 herein (*vide infra*).

Figure 11. (a) Structures and (b) synthesis of base-linker-substrate conjugates **8**, **9** and **10**.

Figure 12. Covalent attachment of substrate to the pIII protein on phage. (a) DNA encoding the acid peptide sequence and a C-terminal cysteine was fused to the N-terminal end of gene gIII, to form the acid helper phage. A phagemid encodes the protein library in fusion with the pIII protein; (b) Phage production leads to phage particles displaying the phagemid encoded protein; the pIII proteins have acid peptide extensions; (c) Coiled-coil formation of the acid and base peptides noncovalently attaches the substrate to the phage pIII protein; (d) Removal of the reducing agent leads to crosslinking of acid and base peptides through their C-terminal cysteines; (e) In the present study phages displaying staphylococcal nuclease are attached to streptavidin beads through a 5'-biotinylated, single-stranded oligodeoxynucleotide. Phages displaying active enzyme are released by cleavage of the oligodeoxynucleotide in an intramolecular reaction.

Figure 13. Immobilization and cleavage of phage from solid support. Either no base-linker, the base-linker-pTp or the base linker-oligodeoxynucleotide conjugate was crosslinked to (a) phage displaying SNase or (b) the control protein Fab 39-A11. Columns 1-4 show immobilization on streptavidin beads. Immobilization was either examined by phage titering of the beads directly (columns 1-3), or after DNase I treatment of the beads (column 4); columns 5-7 show leakage (release in absence of Ca^{2+}); columns 8-10 shows Ca^{2+} induced release (cleavage). The per cent recovery is shown in parantheses above the columns.

Figure 14: A figure supporting the description in working example 2 herein (vide infra).

Embodiment(s) of the present invention is described below, by way of examples only.

DETAILED DESCRIPTION OF THE INVENTION:

A method for multiple catalytic activity turn-over *in vitro* selection, according to the first aspect of the invention:

The term "selection" preferably denotes that the selection according to step (ii) in the first aspect of the invention, is performed on more than 1000 individual units comprised within a sample, preferably without interference of the skilled person.

The term "Column" denotes herein all kinds of solid support. Examples are: columns, surfaces including biacore apparatus. Further In some cases there is no need for a solid support. This is the case if the separation is based on migration in an electric field.

The product generated by a catalyst of interest are in contact with one or more reagent(s) which convert it back into the substrate:

As described above, the term "said sample is further under conditions wherein the product generated by a catalyst of interest are in contact with one or more reagent(s) which convert it back into the substrate", according to step (i) of the first aspect of the invention, denotes any reagent(s) and any combination of reagent(s) molecules which can catalyses the reaction product (comprised within the individual unit) (P_1) to the substrate (comprised within the individual unit) (S_1) or that participates as a reactant in the reaction P_1 to S_1. For further description of this and description of preferred embodiment of said reagent(s) reference is made to the section "Guidance to a skilled person when searching for a suitable reagent" below. Further, see figure 3 for a graphic illustration.

For illustration as a non-limiting example the it is preferably in cases wher the reagent(s) cannot efficiently act on the immobilized product, but only on the free product, the affinity of the column for the product must be adjusted to establish an appropriate equilibrium between the unbound and bound product. However, the optimal selection stringency is obtained if the reagent(s) act on both free product and product immobilized on column.

Examples of suitable "reagent(s) are given below:

"Desired reaction", "Reagent(s)":

#1. "DNA polymerization", "DNase"

#2. "RNA polymerization", "RNase"

#3. "RNA polymerization using nucleotides containing unnatural bases", "RNA backbone cleaving enzyme"

#4. "Glycogen degradation", "UDP-glucose + glycogen synthase"

#5. "Polysaccharide synthesis", "Polysaccharide cleaving enzyme"

#6. "Sequence specific dsDNA cleavage", "Sequenase + deoxyribonucleoside-triphosphates"

#7. "Ester hydrolysis", "activated nucleophile"

#8. "Amid bond formation", "protease"

#9. "Lipid hydrolysis", "acetylCoA + "lipid synthase"

Guidance to a skilled person when searching for a suitable reagent:

The conversion product to substrate molecule is in this paragraph termed "direct reloading" or "substrate reloading".

"Conditions" are in this paragraph defined in the context of the direct reloading protocol set-up, i.e., under conditions where substrate S_1 and product P_1 are attached to the individual unit comprising the catalyst as described above. Further see figure 5 for an illustration of this S_1, P_1, S_2, P_2 definition.

A catalyst cannot alter the equilibrium of a chemical reaction. In other words, a catalyst accelerates the forward and reverse reaction by the same factor. However, under conditions where the reactants have not yet reached equilibrium, a catalyst can accelerate the attainment of equilibria.

In order for the direct substrate reloading protocol to work optimally, the reaction catalyzed by the "catalyst" (individual unit) preferably is energ tically favorable, *i.e.*, the catalyzed (target) reaction is energetically downhill under the conditions of the assay (see Figure 5, A-D). The following examples describe some of the many possible combinations of "target reactions" and "reagent(s)" that might be feasible with the direct reloading protocol. The characteristics of reagent(s) that work is described in each case, in order to guide the skilled person to the right choice of reagent(s).

Figure 5 A depicts a reaction involving only one substrate (S_1) and one product (P_1), both attached to the "catalyst". An example of one such reaction could be the isomerization of a compound (S_1) to its isomer (P_1). The conversion of S_1 to P_1 is energetically downhill, and thus is a reaction that can be targeted with the protocol proposed. The reverse reaction, conversion of P_1 to S_1 would be energetically uphill; therefore, the buffer must contain a reactant that, together with P_1, is at a higher energy than S_1. An example of one such reactant is ATP; in the example, the buffer would contain both ATP and an enzyme that could use the energy stored in ATP to isomerize P_1 to S_1 (ATP would not necessarily react with P_1, but rather, the enzyme would couple the energetically unfavourable conversion of P_1 to S_1 with the energetically favourable hydrolysis of ATP).

Figure 5 B shows a forward reaction involving more than one substrate but only one product. An example would be RNA polymerization, in which the S1 substrate was the 3'-hydroxyl group on a ribonucleotide, and the S2 substrates were various ribonucleoside-triphosphates in solution. The forward reaction would be driven by hydrolysis of the ribonucleoside-triphosphates, and therefore be energetically downhill. However, the substrate reloading process (regeneration of the 3'-hydroxyl) would also be downhill. At a first glance this may seem odd; however, the two processes proceed along different reaction pathways (for example, the ribonucleoside-triphophates are not regenerated). In principle, the reverse reaction does not require an enzyme. However, in the example the reverse process is a hydrolysis of a

phosphodiester bond, which is a very slow process. Substrate reloading would therefore be much more efficient if a ribonuclease was added.

Figure 5 C involves one substrate S1 and two or more products P1 and P2, of lower energy than S1. Reactant is added to the buffer; the reaction of reactant with P1 to form S1 is energetically downhill, as it must be for the set-up to work. This set-up could be used for "catalysts" (individual units) that catalyzed the hydrolysis of glycogen (i.e., the S1 substrate would be glycogen), and therefore the P1 product attached to the catalyst would be a (polymer of) glucose. The reagents could include the enzyme glycogen synthase, which generates glycogen from UDP-glucose and the (polymer of) glucose. The enzyme thus couples the energetically favourable hydrolysis of UDP-glucose with the energetically unfavourable fusion of glucose units.

Figure 5, D describes a more complex situation, in which more than one substrates is transformed into more than one products. Again, the formation of products is down the energy hill; the figure describes a situation where formation of S1 from P1 is spontaneous (does not require a reactant). If the target reaction is an ATP-dependent isomerization (thus, S2=ATP and P2=ADP), and S1 and P1 are isomers, then the isomerization of P1 to S1 might be energetically downhill (and not require any additional reactant). The efficiency of the substrate reloading process could be speeded up by addition of another catalyst (enzyme) catalyzing the ATP-independent isomerization.

It is important to remember that water molecules could be regarded as substrates in for example hydrolysis reactions.

Also, despite what is said above, the direct reloading selection can be set up in a way so that the target reaction is *not* thermodynamically downhill under the conditions of the assay. Consider for example a target reaction where S1 is cleaved to form P1 and P2, and an excess of P2 is present in the buffer so that formation of S1 from P1 and P2 is energetically favoured, *i.e.*, the equilibrium is towards formation of S1. However, since the equilibrium is dynamic, S1 will continuously be degraded and formed, but at any time there will be more S1 than P1. When the individual event S1 to P1 and P2 occurs, the P1 may be isolat d

(eg., bound to a product-binding column), and therefore, the catalyst will be isolated with it. The excess P2 will result in a very efficient substrate reloading, but will not exclude the forward reaction from occuring.

Preferably, the reagent(s) should regenerate the substrate S1 attached to the catalyst.

Preferably, the reagent(s) regenerate the substrate by a reaction pathway different from the pathway that generated the product.

A sample comprising a number of different individual units:

As specified above said sample may comprise at least two different individual units and up to numerous different individual units.

The actual number of different individual units generally corresponds to the actual size of the library of catalyst molecules.

Beside said specified different individual units said sample may in principle also comprise any other suitable material.

Further said different individual units comprised within said sample may be dissolved in any suitable buffer, such as water.

Different individual units:

As described above an individual unit comprises the general structure:

a catalyst - a substrate; or

if the substrate has been converted into the product the general structure:

a catalyst - a product.

Further, the term "different individual units" denotes different individual units each independently comprising different catalyst molecules, *i.e.* an example of two different individual units may be

(1) catalyst molecule^1 - substrate; and

(2) catalyst molecule^2 - substrate;

wherein catalyst molecule[1] and catalyst molecul [2] denotes two different catalyst molecules.

Further, "an individual unit" as described herein denotes an individual unit wherein it is possible to physically separate said individual unit from the other different individual units, within said sample, in order to be able to isolate the separate individual unit.

A biologically amplifiable individual unit:

An embodiment of the invention relates to a sample comprising a number of different individual units according to the invention, wherein said individual units of point (i) according to the first aspect of the invention is a biologically amplifiable individual unit and both said substrate and said catalyst molecule are attached on the surface of said biologically amplifiable individual unit.

The term "a biologically amplifiable individual unit" denotes that within said individual unit either;

(i) the catalyst molecule of interest is a biologically amplifiable molecule; or

(ii) the catalyst molecule of interest is biologically encoded by the information comprised within the entity allowing the unambiguous identification of the catalyst molecule;

providing the possibility of amplifying said catalyst molecule of interest in order to obtain multiple copies of said catalyst molecule.

The term "biologically encoded" in point (ii) above denotes that the information is comprised within a DNA or RNA molecule in the form of the genetic codons.

An example of an biologically amplifiable individual unit in relation to point (i) above is an individual unit wherein the catalyst molecule of interest is a DNA or a RNA molecule, since it is well known in the art that DNA or RNA molecules may easily be amplified.

An example of an biologically amplifiable individual unit in relation to point (ii) above is an individual unit wherein the catalyst molecule of interest is a peptide or a polypeptide

and wherein said entity comprising information allowing the unambiguous identification of the catalyst molecule is a DNA molecule encoding said peptide or polypeptide.

In relation to the second example above, a physical connection must exist between the peptide and the DNA that encodes it, in order to isolate the DNA with the peptide it encodes. The connection can be either direct, in which case the peptide is attached directly to the nucleic acid that encodes it, or indirect, in which the peptide is attached to the surface of for example a cell that contains the nucleic acid encoding it. Such a cell is herein termed a "carrier system" as will be further discussed below.

Flexible linker:

An embodiment of the invention relates to a sample comprising a number of different individual units according to the first aspect of the invention, wherein said individual unit of point (i) comprises following structure: catalyst molecule - flexible linker - substrate.

The term "flexible linker" refers herein to the molecules as a whole connecting the catalyst with substrate. For example, if the substrate is attached to a bead through a flexible molecule, and the catalyst is also attached to the bead through a flexible molecule, "flexible linker" will refer to "flexible molecule-bead-flexible molecule", and the characteristics of the flexible linker will reflect the individual characteristics of the two flexible molecules and the portion of the bead that connects the two. Flexible linkers may for instance consist of flexible polypeptides, polyethylen glycol (PEG), and other polymers of reasonable flexibility.

Further, a flexible linker may also connects the catalyst molecule and a carried system (see below).

Carrier systems

An embodiment of the invention relates to a sample comprising a number of different individual units according to the invention, wherein said individual unit of point (i) in the first aspect of the invention comprises the following structure:

catalyst molecule - carrier system - substrate, or more preferably the structure: catalyst molecule - carrier system - flexible linker - substrate.

The term "carrier system" denotes a system/identity which physically connects the catalyst molecule and the substrate and wherein said carrier system does not directly participate in the catalytic reaction substrate to product catalysed by the catalyst molecule.

Such a carrier system is herein further divided into a biologically amplifiable carrier system and a biologically non-amplifiable carrier system.

Examples of biologically amplifiable carrier systems include (carrier system - catalyst molecule): phage - polypeptide, filamentous phage - peptide, plasmid - peptide, polysome - peptide, bacteria - peptide, and mRNA - peptide.

Examples of biologically non-amplifiable carrier systems include
(carrier system - catalyst molecule): bead - organic molecule, pin - inorganic molecule, and bead - DNA sequence.

It should be noted that an individual unit comprising the beads - DNA sequence structure is herein a biologically amplifiable unit (se above), however the carrier system, as such (bead) is a biologically non-amplifiable carrier system.

Catalyst and library of catalyst molecules:

As stated above the term "catalyst" denotes any catalyst molecule with a desired catalytic activity, such as organic and inorganic molecules, proteins, peptides, nucleic acids, biopolymers and non-biological polymers, small organic or inorganic molecules. Further the terms "catalyst" and "catalyst molecule" may be used interchangeably.

Accordingly, further embodiment of the invention relates to,

(i) a sample comprising a number of different individual units according to the invention, wherein said library of catalyst molecules is a library of peptides or polypeptides, preferably a library of enzymes;

(ii) a sample comprising a numb r of different individual units according to the embodiment (i) immediately above, wherein said library is a library comprising polypeptides individually having a number of different enzymatic activities; or

(iii) a sample comprising a number of different individual units according to the embodiment (i) above, wherein said library is a library comprising polypeptides variants derived from one or more precursor polypeptide(s), wherein said precursor polypeptide(s) exhibit(s) closely related enzymatic activities.

The term "library comprising polypeptides individually having a number of different enzymatic activities" preferably denotes a ~~library wherein said different enzymatic activities~~ are substantially different activities, e.g. protease, amylase, xylanase, cellulase activities. An advantage of such an library may be that by chancing the substrate according to the specific activity of interest, said library may be used to identify a number of polypeptides of interest. If for instance a protease of interest first is isolated by a method for in vitro selection as described herein by use of e.g. a peptide as substrate, then an amylase may be isolated thereafter by chancing the substrate to a e.g. a starch molecule.

Said libraries may be made according to any of the numerous standard known processes of making such libraries.

Accordingly, an further embodiment of the invention relates to a sample comprising a number of different individual units according to the embodiments of invention mentioned immediately above, wherein said library of polypeptides is a library comprising shuffled/recombined polypeptides.

Further embodiments of the invention relates to

(i) a sample comprising a number of different individual units according to the invention, wherein said library of catalyst molecules is a library comprising natural polymers molecules, or unnatural polymers molecules, or small organic molecules, or small inorganic molecules or a mixture of said molecules; or

(ii) a sample comprising a number of different individual units according to th embodiment mentioned immediately above, wherein said library is made by combinatorial chemistry.

Preferably, the sample may contain a virtual combinatorial library, in the sense that each potential catalyst is made up of more than one entity, and the entities associate and dissociate several times during the multiple turn-over assay.

Such catalysts could be catalysts made up of several polypeptide chains, held together by weak interactions, and therefore continuously associating and dissociating.

Alternatively, the library members could be assemblies of small organic molecules held together by disulfide bonds.

In an even further embodiment the invention relates to a sample comprising a number of individual units according to the invention, wherein the catalyst molecules and the substrate capable of being catalysed into a product (point (i) in the first aspect) are of a different chemical substance.

The term "catalyst molecules and the substrate capable of being catalysed into a product (point (i) in the first aspect) are of a different chemical substance" denotes that said catalyst molecule and the substrate molecule are of a substantial different chemical substance.

Accordingly, in this preferred embodiment the situation wherein the catalyst molecules and the substrate molecule are both DNA or RNA molecules are herein said to be a situation wherein the catalyst molecules and substrate molecules are NOT of a different chemical substance.

Further, an embodiment of the invention relates to a sample comprising a number of individual units according to the invention, wherein the catalyst molecules is NOT a nucleic acid molecule such as DNA or RNA.

Means of isolating an active catalysts of interest according to a method of the invention:

The separation of active and less active catalysts

preferably involve a selective step during which the catalyzed reaction leads to either release or attachment of the catalyst through the linker-substrate attached to it.

There are principally four means to separate active from inactive catalysts. i) The active catalysts can be isolated by immobilization of the product on a product binding column (or more generally, by means of the attached product). ii) The inactive catalysts can be removed by immobilization on a substrate binding column. iii) Prior to the target reaction the catalyst may be attached to support; when a cleavage reaction occurs, the catalyst is released from support and can be collected. iv) The active catalysts may attach themselves to solid support upon reaction of substrate 1 (attached to the catalyst) and substrate 2 (attached to support).

The product and substrate specific columns may immobilize the product and substrate through binding to a receptor molecule with specificity for the product and substrate, respectively. Alternatively, immobilization may be mediated by a product- or substrate-specific reaction between functional groups on the column and the product or substrate attached to the catalyst.

Other means of isolating the product or substrate (and with these, the active or inactive catalysts, respectively) include partitioning between different phases, mass spectrometry, precipitation, electric or electromagnetic separation etc.

Accordingly, embodiments of the invention relate to

(i) a method for *in vitro* selection according to the first aspect of the invention, wherein the selecting for a catalyst molecule of interest, in step (ii), is done by specific immobilization to said product molecule;

(ii) a method for *in vitro* selection according to the first aspect of the invention, wherein the selecting for a catalyst molecule of interest, in step (ii), is done by the following strategy,

(a) constructing a system wherein substantially each of the individual units in step (i) of the first aspect comprising the substrate molecule and the catalytic molecule is bound to a matrix and

wherein the unit is released from said matrix when the substrate is converted into the product; and

(b) selecting for the unit(s) which are released from said matrix; or

(iii) a method for *in vitro* selection according the first aspect of the invention, wherein the selecting for a catalyst molecule of interest (step (ii)), is done by the following strategy,

(a) constructing a product-column wherein a receptor specifically binding the product is placed along the matrix of the product-column; and

(b) adding the sample of individual units at one end of the product-column and selecting for the catalyst molecules of interest by isolating the individual unit(s) which arrive(s) latest to the opposite end on the column.

Repeating step (i) to (iii) one or more times, according to point (iv) of the first aspect of the invention:

As stated above the term "repeating step (i) to (iii) one or more times by using the information comprised in said entity of step (iii) to generate the catalyst molecule of interest and construct an individual unit comprising said generated catalyst molecule of interest and then using this individual unit as a starting material in said repetition step" according to point (iv) in first aspect of the invention denotes that said repetition may be one time, more preferably 2 times, more preferably more than 5 times, even more preferably more than 10 times, and most preferably more than 25 times.

A method for producing a catalyst molecule of interest, according to the final aspect of the invention:

As stated above, in a final aspect the invention relates to a method for producing a catalyst molecule of interest comprising performing the method *in vitro* selection according to the invention and the further following steps,

(a) producing said isolated catalyst molecule of interest in a suitable quantity of interest by a suitable production method.

As described above, in the method for *in vitro* selection, according to the first aspect the invention step (iii) reads:

"(iii) isolating an entity comprising information allowing the unambiguous identification of the catalyst molecule of interest which has been capable of catalysing multiple times the reaction substrate to product, by means of a characteristic of the product."

Accordingly, the information comprised within said entity provides the possibility of producing said catalyst molecule of interest by any standard production strategy known to the skilled person.

If said catalyst molecule of interest for instance is a polypeptide of interest said standard production strategy may be a standard protocol for recombinant production of said polypeptide of interest, or

if said catalyst molecule of interest for instance is an organic molecule of interest said standard production strategies may be a standard protocol for production of such an organic molecule.

EXAMPLES:

Example 1:

An example of an individual unit comprising the features of the first aspect of the invention.

In this example 1, the catalyst of interest is a SNase; substrate is a single stranded oligonucleotide (ssDNNA); and product is the ssDNA cleaved by a SNase of interest.

Further, a filamentous phage is used as a carrier system and an acid/base linker is used as a flexible linker.

Accordingly, the individual units in this example has following general structure:

SNase - fil. Phage - acid/base link. - ssDNA

Catalyst - Carrier system - flexible linker - substrate.

See figure 12 for an illustration.

In this example the "selection characteristic" of the product (i. . cleaved ssDNA) is that said product does not bind to a matrix and the substrate (ssDNA) does bind to a matrix.

Accordingly, in this example a SNase molecule of interest is isolated by selecting for individual units which are released from said matrix. See figure 12 for an illustration.

MATERIALS AND METHODS.

Synthesis of compounds. Fmoc-S-(2-nitro-4,5-dimethoxybenzyl)-L-cysteine **1** was synthesized by a variation of the method of Merrifield (6). Briefly, 605 mg L-cysteine (5 mmol) was suspended in 100 mL degassed ethanol/water (2:1), and 1.39 mL triethylamine (10 mmol) and 1.39 g 1-(bromomethyl)-2-nitro-4,5-dimethoxybenzene (5 mmol) were added. The mixture was stirred for 10 h at 23 °C in the dark under nitrogen and filtered. The filter cake was washed with ethanol and recrystallized from ethanol/water to provide 0.95 g S-(2-nitro-4,5-dimethoxybenzyl)-L-cysteine (3 mmol). The recrystallized product (0.8 g) was suspended in 20 ml water; 0.53 ml triethylamine (3.8 mmol) was added followed by a solution of 0.9 g 9-fluorenylmethoxycarbonyl succinate ester (2.7 mmol) in 12 mL acetonitrile and the mixture stirred for 10 h at 25 °C under nitrogen. The product precipitated upon acidification to pH 2-3 with 1 M HCl and evaporation of the acetonitrile. The precipitate was collected on a frit and washed with water and ethylacetate to remove excess HCl and reagent. The resulting crude product **1** (1.13 g) was extensively dried under vacuum, and used directly in the synthesis of the base-linker peptide C(GGS)$_4$AQLKKKLQALKKKNAQLKWKLQALKKKLAQGGC (base sequence underlined, photoprotected cysteine in bold). Compounds **2**, **3** and **4** were synthesized on an ABI DNA synthesizer on a 1 mmole scale with a 3'-biotin group (BiotinTEG CPG, Glen Research) and a 5'-thiol (5'-Thiol-Modifier C6, Glen Research) and purified by reverse phase HPLC following removal from the resin (Rainin Microsorb C18 column, flow 1 mL/min.; solvent A: 50 mM triethylammonium acetate (TEAA), pH 7, solvent B: acetonitrile, linear gradient from 5 to 50 % solvent B over 40 min); the trityl protecting group on th thiol was removed according to the protocol of Glen Research. The products were

lyophilized and dissolved in water (1.0 mM final concentration). The conjugate of **2** with the base-linker peptide was prepared as follows: 2 mg (415 nmole) base-linker peptide was reacted with a 20 fold molar excess of N,N'-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (3.2 mg) in 1 mL of 50 mM sodium phosphate buffer, pH 5.5, for 10 h under nitrogen at 4 °C. Compound **5** was purified from the reaction mixture by reverse phase HPLC (Vydac RP-18 column, flow 2 mL/min; solvent A: 0.1 % TFA in water, solvent B: 0.1 % TFA in acetonitrile; linear gradient from 10 to 55 % solvent B over 35 minutes), and the product fractions concentrated to approximately 0.3 mL (OD_{280} = 6, compound **5** should not be concentrated to dryness). To 100 mL (138 nmoles) of this solution was added 75 ul water, 75 mL of aqueous 1 M aqueous sodium phosphate, pH 7, 30 mL of aqueous 5 M NaCl, and 22 mL (22 nmoles) of compound **2**, and the reaction incubated for 10 h under nitrogen at 23 °C (to avoid precipitation the reagents should be added in this order). The product was purified by anion exchange FPLC (Mono Q HR 5/5 column (Pharmacia), flow 0.75 mL/min solvent A: 20 mM Tris-HCl, pH 7, solvent B: 20 mM Tris-HCl, pH 7, 2 M NaCl; linear gradient from 20 to 60 % B in 7.5 min); on a 10 % denaturing polyacrylamide gel the product ran as a single band. Fractions of OD_{260} = 0.3-1 were used directly for the photo-deprotection step (*vide infra*). The conjugates of **3** and **4** with the base-linker-peptide were prepared as follows: approximately 200 nmoles of either **3** or **4**, and a 20 fold excess of bismaleimide were incubated in 1 mL of aqueous 50 mM phosphate buffer, pH 5.5, at 4 °C for 15 hours. After purification by reverse phase HPLC and lyophilization, the identity of compounds **6** and **7** was verified by Maldi-ToF MS. Either **6** or **7** (150 nmoles) was then incubated with 100 nmoles base-linker-peptide in 100 mL of 10 mM TEAA, pH 6.5, 100 mM NaCl for 15 hours at 4 °C. The products were purified by reverse phase HPLC (Vydac RP-18 column, conditions as described above), lyophilized and analyzed by Maldi-ToF MS (7). The 2-nitro-4,5-dimethoxybenzyl protecting group on the C-terminal cysteine of the three conjugates was removed by photolysis to afford compounds **8**, **9** and **10** as follows: for compound **8**, 100 mL of the FPLC purified fraction containing the protected conjugate (*vide*

supra) was degass d thoroughly with argon for 15 min, and then exposed to a mercury lamp (450 W high pressure mercury lamp, Ace-Hanovia; Pyrex™ filter, cutoff ² 300 nm) in a septum capped glass vial for 30 min (8). For compounds **9** and **10**, 10 nmole of the conjugate was dissolved in 100 mL of 10 mM DTT, degassed and photolyzed as described above. After 30 min of irradiation no remaining starting material could be detected by MALDI-ToF MS. The reaction mixture was separated by HPLC (Vydac RP-18 column, conditions as described above) and the product fractions were lyophilized. The conjugates were stored frozen, and used within a week after photo-deprotection, to ensure efficient attachment to phage.

Construction of acid helper phage. A NarI restriction site was introduced between the third and fourth codon of mature pIII protein of M13K07 helper phage (Promega) by Kunkel mutagenesis (9) with the primer K07-NarI-prim (5'-ACAACTTTCAACGGCGCCAGTTTCAGCGG-3') to give NarI-helper phage. DNA encoding the amino acids GAAQLEKELQALEKENAQLEWELQALEKELAQ-**GGC**PAGA (acid peptide sequence underlined, GGC motif in bold) with a NarI restriction site at both ends, was produced by polymerase chain reaction (PCR) with the plasmid pCRII acid (Ellis L. Reinherz, Dana Farber Cancer Institute, Boston) with th primers NarIfwd (5'-ACTACAAATTGGCGCCGCTCAGCTCGAAAAAGAGC-3') and NarIbck (5'-AATTATAGGCGCCAGCCGGGCAACCGCCCTGAGCCAGTTCCTTTTCC-3'). The PCR product was digested with NarI and inserted into NarI digested NarI-helper phage to afford acid helper phage.

Construction of phagemids encoding the staphylococcal nuclease-pIII fusion and 39-A11 Fab-pIII fusions. To make the SNase-pIII fusion, PCR was performed on the plasmid pONF1 (10), carrying the gene encoding SNase, with primers 5'-CGCGAATTGGCCCAGCCGGCCATGGCCGCAACTTCAACTAAA-3' (SfiI restriction site underlined) and 5'-GCGAATTGGTGCGGCCGCTTGACCTGA-ATCAGCGTTG-3' (NotI restriction site underlined). The product was digested with SfiI and NotI and inserted into SfiI-NotI digested pFAB-5c.His, a derivative of plasmid pFAB-5c (11), to give phagemid pII78-6. As a negative control the phagemid pComb3H.DA was employed. This phagemid (12) carries the 39-A11 Fab antibody (13)

fused to the pIII protein. The expression of both the SNase and control protein is driven by the *lac* promoter.

Production of phage particles. Phage particles were produced with minor modifications according to Ørum *et al.* (11). Briefly, *E. coli* XL1-blue was transformed with pII78-6 or pComb3H.DA, and shaken at 37 °C in 2xYT broth and 100 mg/mL ampicillin. At an OD_{600} of 0.5, acid helper phage was added to a final concentration of 1.5 x 10^8 cfu/mL, and incubated at 37 °C for 20 min. The cells were pelleted and resuspended in 2xYT, 100 mM IPTG, 100 mg/mL ampicillin, 50 mg/mL kanamycin, and shaken for 14 hours at RT. Cells were pelleted and phage particles in the supernatant were PEG precipitated, followed by resuspension in TBS (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2.5 mM KCl). Phage titrations were performed with *E. coli* XL1-blue using standard procedures (14).

Covalent attachment of base-linker-substrate conjugates to phage.

Approximately 10^8 phage particles were incubated in 40 mL buffer A (TBS, 10 mM EDTA, 0.1 % BSA), supplemented with 1 mM mercaptoethylamine (MEA) and 1 nmole of either base-linker-oligodeoxynucleotide (**8**), base-linker-pTp (**9**) or base-linker-pTpTp (**10**), at 37 °C for 60 minutes, then PEG precipitated twice and resuspended in buffer A.

Phage immobilization and release from solid support. Approximately 10^8 phage particles, covalently attached to the base-linker-substrate conjugates, were incubated with 50 mL magnetic streptavidin beads (Boehringer Mannheim, biotin binding capacity: 1.5 nmole/mL) in 1 mL buffer A for 15 minutes at 23 °C; eight 1 min washes were performed in buffer A with 0.1 % Tween 20, followed by two 1 min washes in buffer A. The number of phage immobilized on the beads was determined by suspending the beads in buffer A, and then either directly infecting *E. coli* XL1-blue with the bead suspension and titering or alternatively, infecting after treatment of the beads with DNase 1 (1unit/mL DNase 1, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 8, 23 °C for 15 min). Calcium-dependent release (cleavage) from solid support was examined by suspending beads in buffer B (TBS, 10 mM $CaCl_2$, 0.1 %

BSA), incubating at 23 °C for 5 min, and titering the supernatant. Calcium-independ nt release from the beads (leakage) was determined by resuspending the beads in buffer A, incubating for five minutes at 23°C, and titering the supernatant.

Enrichment of active enzymes from a library-like ensemble. Phage particles displaying SNase or 39-A11 Fab were mixed in a 1:100 ratio and the base-linker-oligodeoxynucleotide conjugate (**8**) was covalently attached. Phage were then immobilized on magnetic streptavidin beads, washed in buffer A, and incubated in buffer B as described above, *E. coli* XL1-blue were infected with the supernatant and the cells plated on a LA plate containing 100 mg/mL ampicillin. Randomly picked colonies were identified as SNase- or control clones by PCR or restriction enzyme digestion.

RESULTS & DISCUSSION.

Selection scheme. To test the above strategy for directed-enzyme evolution in a phage-display format, it was first necessary to develop a general method for selectively attaching a given substrate to or near a phage-displayed enzyme. Importantly, the substrate must be attached so that it can bind productively in the active site of the conjugated enzyme. Moreover, the substrate should be covalently linked to the phage to ensure that there is no crossover of reaction product between members of the library. One possible strategy involves selective chemical modification of the enzyme or a nearby phage coat protein (e.g., pIII protein) with substrate by a disulfide exchange reaction. For example, a cysteine residue introduced near the active site of staphylococcal nuclease through site-directed mutagenesis has been used to selectively introduce unique chemical functionality by a disulfide exchange reaction (15). To apply this method to proteins expressed on filamentous phage, the three single cysteines of the pVI, pVII and pIX coat proteins were first mutagenized to alanine. The eight buried cysteine residues in the pIII protein were left unchanged, as they likely form structurally important disulfide bridges (16). Unfortunately, repeated attempts to selectively modify unique cyst ine residues introduced near the active site of several enzymes displayed on phage, by either disulfide exchange,

maleimide addition or alkylation reactions, resulted in significant nonspecific labelling of phage coat proteins. No conditions or reagents were found that made possible selective labelling of the pIII fusion protein containing the unique surface cysteine residue. It is likely that the thousands of proteins constituting the phage coat make the specificity requirement for a chemical reaction too great; also, the probability of cysteine misincorporation due to the intrinsic error rate in protein biosynthesis becomes significant for such a large ensemble of proteins. Alternatively, the cysteine residues in the pIII protein may be accessible to crosslinking reagents.

To circumvent these problems, a two step process was developed in which chemical crosslinking is preceded by the selective formation of a noncovalent complex at the site of modification (Figs. 11 and 12). The complex is a heterodimeric coiled-coil consisting of a synthetic basic peptide **B** C(GGS)$_4$AQLKKKLQALKKKNAQLKWKLQALKKKLAQGGC, to which substrate is covalently coupled before heterodimerization, and an acidic peptide **A**, GAAQLEKELQALEKENAQLEWELQALEKELAQGGCPAGA that is expressed as an N-terminal fusion to the pIII coat protein of filamentous phage. The acid and base peptides (underlined) were chosen as dimerization domains because of their small size (thirty amino acids) and high tendency to form stable, parallel heterodimeric coiled-coil structures -- the acid-acid and base-base homodimers form 10^5 fold less efficiently than the heterodimer (17). Heterodimerization of the synthetic (**B**) and phage-encoded (**A**) peptides should bring the substrate into close proximity of the displayed enzyme, and lead to spontaneous disulfide bond formation between cysteines on each of the peptides (Fig. 12). The tripeptide Gly-Gly-Cys was added to the C-termini of the acid and base peptides to facilitate formation of a disulfide bridge between the two helices (17). The substrate is covalently linked to the basic peptide **B** through a flexible linker to facilitate productive binding of substrate to enzyme (Fig. 11). The acidic peptide **A** is fused to the pIII protein of the phage rather than to the displayed enzyme itself for the following reasons: (i) insertion of the acid peptide sequence into an enzyme might interfere with enzyme function; (ii) the

flexible linker of the base-linker-peptide as well as hinges in th pIII protein and a peptide linker inserted between pIII and the displayed enzyme, should allow many possible orientations of the substrate relative to the enzyme active site; and (iii) it should be possible to use a single helper phage bearing the acid peptide extension to display many enzyme-substrate pairs, rather than having to engineer into each enzyme a functional conjugation site.

Generation of the acid helper phage and base-linker-substrate conjugate.

To attach the base-linker-substrate conjugate to phage we introduced the acidic peptide **A** at the N-terminus of pIII protein in the M13K07 helper phage. The enzyme library is fused to the N-terminus of the pIII coat protein; this construct is carried in the phagemid. Upon superinfection by helper phage, phage particles are produced that contain the phagemid DNA but whose coat consists (with one exception) of proteins encoded by the helper phage genome. The one exception is the pIII protein, present in 4-5 copies at one tip of the phage. During packaging of the phage, both enzyme-pIII fusions and acid peptide A-pIII fusions are produced; the phage particles obtained from a typical preparation carry either one or zero enzyme-pIII fusions plus three to five copies of acid peptide A-pIII fusion.

To generate phages bearing an acid peptide-pIII fusion, DNA encoding the acidic peptide A with a C-terminal extension containing a cysteine residue, was introduced into the 5'-end of gene III of the M13K07 helper phage. The resulting acid helper phage particles were immoblized more than hundred fold more efficiently than M13K07 on an ELISA-plate coated with basic peptide **B**, indicating that the mutant helper phage carry accessible acid peptide extensions on their pIII proteins. Likewise, when *E. coli* containing a phagemid encoding a pIII fusion protein were superinfected with the acid helper phage, the resulting phage particles displayed modified pIII extensions in addition to the pIII fusion protein (Fig. 12). The insertion of the acid peptide did not appear to change the titer or rescue efficiency of the helper phage significantly.

The synthetic base-linker-peptide (B) to which substrate is attached consists of the twelve residue $(GlyGlySer)_4$ linker followed by the thirty amino acids constituting the base sequence (Fig. 11). The base-linker peptide also contains cysteine residues at the N-and C-termini that allow efficient, selective coupling of the peptide to substrates and disulfide bond formation to phage, respectively (Figs. 11 and 12). The C-terminal cysteine of the synthetic peptide is initially protected with the photochemically removable 2-nitro-4,5-dimethoxybenzyl protecting group. This allows substrate to be selectively conjugated by a thiol specific reaction (e.g., by disulfide exchange, alkylation, or Michael addition reactions) to the free thiol group of the N-terminal cysteine. After substrate conjugation, the C-terminal cysteine is photochemically deprotected in high yield to generate a free thiol available for crosslinking to the acid peptide extension on phage. Because the chemical conjugation of substrate and base-linker peptide, and the crosslinking of this conjugate to phage are carried out separately, many different chemistries and reaction conditions can be used to couple the base-linker peptide and substrate. Moreover, the composition of the conjugate can be purified and characterized (e.g., by mass spectrometry) before it is crosslinked to phage.

Staphylococcal nuclease as a model system. The enzyme staphylococcal nuclease is a well-characterized enzyme consisting of a single polypeptide chain 149 amino acids in length (18). The enzyme preferentially hydrolyzes the phosphodiester bonds of single-stranded RNA (ssRNA), ssDNA, and duplex DNA at A,U- or A,T- rich regions to generate 3'-phosphate and 5'-hydroxyl termini (18). Ca^{2+} is required for enzymatic activity, providing a mechanism for modulating enzyme action. In addition, SNase has successfully been displayed as a pIII fusion protein on phage (19).

Because no reagent, antibody or receptor is available that can easily distinguish between a single-stranded oligodeoxynucleotide substrate and its cleavage product (a complementary oligonucleotide would be degraded), a selection scheme was de-

veloped in which enzymatic cleavage of ssDNA substrate results in release of phage from solid support. In this scheme, one round of selection involves the following steps: i) attachment of phage displaying SNase to solid support through a single-stranded oligodeoxynucleotide (in the absence of Ca^{2+} to inactivate SNase); ii) removal of unbound phage by washing; iii) initiation of the cleavage reaction by addition of Ca^{2+}, and iv) isolation of eluted phage. In later rounds of selection, elution can be done under increasingly stringent conditions, eg., shorter reaction time, lower temperature and altered pH. Attachment of phage to solid support is carried out by coiled-coil formation between 5'-biotinylated oligodeoxynucleotide-peptide **B** conjugates and acid peptide **A** extensions on phage, followed by disulfide crosslinking of the two peptides and immobilization on streptavidin beads (Fig. 11). This scheme, in which the phage is attached to solid support through the substrate, requires that the enzyme or substrate be maintained in an inactive state during attachment to phage, and then be activated by a change in reaction conditions. Such changes can include modulation of pH, addition of cofactors or co-substrates, and photochemical or chemical activation of the substrate. In the case of biomolecular condensation reactions in which bond formation results in phage immobilization on solid support, it is not necessary to initiate the reaction; the same is true if capture of active enzymes is by a product-specific reagent, antibody or receptor.

Covalent attachment of the substrate to phage. Phage displaying either SNase or a control protein (antibody 39-A11 Fab fragment) were prepared by superinfection with the acid helper phage. To evaluate the efficiency of the attachment of base-linker-substrate conjugates to phage, an excess of a control conjugate, "pTp"-peptide **B** (compound **9**), was incubated with the phage. The base-linker-pTp conjugate consists of a biotin moiety, followed by deoxythymidine-3',5-diphosphate (pTp), the flexible peptide linker and base peptide sequence, and a C-terminal cysteine. The base-linker-pTp conjugate is not a substrate for wildtype SNase in solution (pTp is a potent inhibitor of SNase) (20). Phage and the substrate-peptide **B** conjugate were first incubated with the reducing agent mercaptoethylamine (MEA) to reduce di-

sulfide bonds between cysteines on the phage acid peptide or the synthetic peptide. Then, MEA and fre base-linker-pTp were removed by PEG precipitation, and magnetic streptavidin beads were added. After ten washes, the number of phage that were immobilized was determined by infection of *E. coli* XL1-blue with the beads, and titering phage. When measured this way, the efficiency of phage immobilization was approximately 10%, for both phage displaying SNase and 39-A11 Fab (Fig. 13).

Next it was determined whether an oligodeoxynucleotide substrate attached to phage displaying SNase would be stable in the absence of Ca^{2+}. The base-linker-oligodeoxynucleotide conjugate was attached to phage displaying SNase (in the presence of EDTA), and the immobilization efficiency determined as above. The efficiency of immobilization was again approximately 10% (Fig. 13), indicating that the tethered oligodeoxynucleotide substrate is not cleaved by SNase in the absence of Ca^{2+}. It is possible that the true immobilization efficiency is higher than observed if some of the phage are rendered non-infective when attached to the beads. This notion was tested by addition of DNase I, which should cleave the tethered oligodeoxynucleotide substrate and release the immobilized phage. As can be seen in Fig. 13, most of the immobilized phage are non-infective, but become infective upon addition of DNase I, indicating that the true immobilization efficiency is about 80 % (Fig. 13). If the oligodeoxynucleotide-peptide **B** conjugate is not included, less than 0.01% of the phage become immobilized; if the wildtype M13K07 helper phage is used to superinfect, about 0.3% of phage are immobilized. It thus appears that the two-step protocol for attachment of substrate to phage pIII protein is efficient and highly site-specific.

Enzyme dependent cleavage of phage from solid support and enrichment. To determine whether phage-displayed SNase is capable of specifically cleaving the tethered oligodeoxynucleotide substate in an intramolecular reaction, Ca^{2+} was added to the immobilized phage to activate the enzyme. Approximately 15 % of the phage were released (Fig. 13), in contrast to release of only 0.2% of the control phage displaying Fab 39-A11 (Fig. 13). This

xperiment demonstrates that SNase cleaves and releases phage from the solid support much more efficiently than the control protein, as expected. However, it appears that a small but significant fraction of the phage leak off the support during the assay (this background leakage is observed without Ca^{2+}, for both the base-linker-oligodeoxynucleotide and base-linker-pTp conjugates, and for both displayed proteins, Fig. 13. Addition of Ca^{2+} leads to an initial burst of phage release from support; however, the release of phage quickly declines to a level corresponding to the leakage observed without Ca^{2+}. This result demonstrates that phage released into solution by intramolecular cleavage events do not release other phage from support as a result of intermolecular cleavage reaction. Cross-reactivity therefore does not appear to be significant, even with a very active enzyme like SNase.

The above analysis suggests that it should be possible to enrich phage displaying SNase from a library-like ensemble of phage displaying catalytically inactive proteins. To test this, phage displaying SNase and the Fab 39A-11 control protein were mixed in a ratio of 1:100, crosslinked to the oligodeoxynucleotide-peptide B conjugate and immobilized. After incubation with Ca^{2+}, the ratio of recovered phage was 22:18, which corresponds to an enrichment factor slightly higher than 100. This degree of enrichment should be sufficient to isolate an active catalyst from a library of 10^{10} members after five rounds of selection and amplification. The enrichment factor can be increased by decreasing background leakage of phage from support. This leakage may result from release of streptavidin from support, or alternatively, reduction or incorrect formation of the disulfide bridge between the synthetic and phage encoded peptides. We are currently exploring these possibilities. Alternatively, the enrichment factor can be increased by increasing the extent of the enzyme-catalyzed cleavage reaction. Under the conditions of phage production, the ratio of pIII expressed from the helper phage relative to the pIII fusion protein expressed from the phagemid is such that most of the phage carry only wildtype pIII

proteins; only a minor fraction of the phage carry the protein-pIII fusion. The number of phage that can cleave themselves off can be increased simply by increasing the number of phage that display the enzyme. For the phagemid/helper phage combination described here, we estimate that only about 15 % of the phage are monovalent. By appropriate vector design and phage preparation, it should be possible to increase the average display to about one protein per phage. This should increase the cleavage to leakage ratio 7 fold, and hence, increase the enrichment factor of active versus inactive enzymes from the present ~100 to about 700.

To examine whether the selection scheme described here can be used for reactions that involve small molecule substrates, a pTpTp-peptide **B** conjugate (compound **10**) was attached to phage displaying SNase or the control protein. Phage were carried through the enrichment routine described above, and again SNase displaying phages were enriched. MALDI-ToF mass spectrometry was used to show that the pTpTp substrate was cleaved at the phosphodiester bond between the two thymidines; no side products were detected. It thus appears that the methodology is applicable to both macromolecular and small molecule substrates. We are currently exploring the possibilities for isolating novel catalysts from libraries of enzyme or antibody origin.

Most enzyme libraries displayed on phage require superinfection by a helper phage like M13K07. The selection protocol described here can therefore be applied directly to these libraries - one simply needs to prepare phage after superinfection of the phagemid encoded library with the acid peptide helper phage, and conjugate the substrate of choice to the basic peptide **B**. Likewise, this methodology can be applied to populations of structurally diverse proteins. The collection of proteins encoded by a genome is one such population. For example, it should be possible to isolate natural kinases with predefined substrate specificity from a genomic protein library using this selection scheme. This type of functional cloning in which a natural enzyme (and the gene that encodes it) is isolated on the basis of its catalytic activity should be applicable to many reactions catalyzed by natural enzymes.

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Example 2:

Optimization of an enzyme with glycosidase activity. This is an example of the selection scheme depicted in Figure 14.

The enzymes with glycosidase activity is displayed on the surface of a filamentous phage using the principles as described in example 1 above and the skilled persons general knowledge.

The substrate is a glykogen linker substrate attached on the surface of a filamentous phage using the principles as described in example 1 above and the skilled persons general knowledge Further, the enzymes are attached to column through the glykogen linker by standard techniques known in the art.

Enzymes with the desired catalytic activity will cleave the bond between the two sugars, releasing the phage with a glucose unit attached to it. However, glycogen synthase present in the buffer will catalyze the condensation of glucose and UDP-glucose, and as a result reattach the enzyme to the column through a portion of glycogen. Enzymes with the highest catalytic efficiency will flow through the column the fastest, and can be collected from the first column fractions.

CLAIMS

1. A method for *in vitro* selection, from a library of catalyst molecules, of a catalyst molecule of interest having a relatively more efficient specific catalytic activity of interest as compared to the rest of the catalyst molecules within said library and wherein said *in vitro* selection method is characterised by that it allows multiple catalytic activity turn-overs (*i.e.* substrate to product catalytic activity turn-overs), by the catalyst molecule of interest, before it is finally collected and wherein said method comprises following steps,

(i) placing; a sample comprising a number of individual units wherein said sample comprises a library of catalyst molecules provided in the form of individual units, wherein the individual units comprise a first type individual unit having the following general structure:

C-S,

wherein C denotes a catalyst molecule and S a substrate which is capable of being catalysed into a product by at least one catalyst comprised within said library of catalyst molecules and thereby providing the possibility of obtaining a second type individual unit comprising the general structure:

C-P,

wherein C has the meaning defined above and P is the product molecule resulting from the catalytic conversion of the substrate S of the first type individual unit; and

(a) the substrate S is attached to the catalyst in a configuration that allows catalytic reaction between the catalyst and the substrate within said individual unit; and

(b) the nature of said attachment of the substrate and the catalyst provides the possibility, by means of a characteristic of the product, of isolating an entity comprising information allowing the unambiguous identification of the catalyst molecule which has been capable of catalysing the reaction substrate molecule to product molecule;

under suitable conditions where a catalyst molecule of interest performs its catalytic activity of interest and where said method is characterised by that said sample is further under conditions wherein the product generated by a catalyst of interest are in contact with one or more reagent(s) which convert it back into the substrate S;

(ii) selecting for a catalyst of interest by selecting for one or more individual unit(s) which comprise(s) the product molecule; and

(iii) isolating an entity comprising information allowing the unambiguous identification of the catalyst molecule of interest which has been capable of catalysing multiple times the reaction substrate to product, by means of a characteristic of the product; and optionally

(iv) repeating step (i) to (iii) one or more times by using the information comprised in said entity of step (iii) to generate the catalyst molecule of interest and construct an individual unit comprising said generated catalyst molecule of interest and then using this individual unit as a starting material in said repetition step.

2. The method according to claim 1, wherein the individual unit of point (i) in claim 1 is a biologically amplifiable individual unit and both said substrate and said catalyst molecule are attached on the surface of said biologically amplifiable individual unit.

3. The method according to any of the preceding claims, wherein said individual unit of point (i) comprises following structure: catalyst molecule - flexible linker - substrate.

4. The method according to any of the preceding claims, wherein said individual unit of point (i) in claim 1 comprises following structure: catalyst molecule - carrier system - substrate, or more preferably the structure: catalyst molecule - carrier system - flexible linker - substrate.

5. The method according to claim 2 and 4, wherein said carrier system of claim 4 within said biologically amplifiable individual unit of claim 2 is a phage.

6. The method according to claim 4, wherein said carrier system is a bead particle.

7. The method according to any of claims 1 to 6, wherein said library of catalyst molecules is a library of peptides or polypeptides, preferably a library of enzymes.

8. The method according to claim 7, wherein said library is a library comprising polypeptides individually having a number of different enzymatic activities.

9. The method according to claim 7, wherein said library is a library comprising polypeptides variants derived from one or more precursor polypeptide(s), wherein said precursor polypeptide(s) exhibit(s) closely related enzymatic activities.

10. The method according to any of claims 7-9, wherein said library is a library comprising shuffled/recombined polypeptides.

11. The method according to any of claims 1 to 6, wherein said library of catalyst molecules is a library comprising natural polymers molecules, or unnatural polymers molecules, or small organic molecules, or small inorganic molecules or a mixture of said molecules.

12. The method according to claim 11, wherein said library is made by combinatorial chemistry.

13. The method according to any of the preceding claims, wherein the catalyst molecules and the substrate capable of being catalysed into a product (point (i) in claim 1) are of a different chemical substance.

14. The method, wherein the catalyst molecules is NOT a nucleic acid molecule such as DNA or RNA.

16. The method for *in vitro* selection according to any of the preceding claims, wherein the selecting for a catalyst molecule of interest, in step (ii) of claim 1, is done by specific immobilization to said product molecule.

17. The method for *in vitro* selection according to any of the preceding claims, wherein the selecting for a catalyst molecule of interest, in step (ii) of claim 1, is done by the following strategy,

(i) constructing a system wherein substantially each of the individual units in step (i) of 1 comprising the substrate molecule and the catalytic molecule is bound to a matrix and wherein the unit is released from said matrix when the substrate is converted into the product; and

(ii) selecting for the unit(s) which are released from said matrix.

18. The method for *in vitro* selection according to any of the preceding claims, wherein the selecting for a catalyst molecule of interest (step (ii) of claim 1), is done by following strategy,

(a) constructing a product-column wherein a receptor specifically binding the product is placed along the matrix of the product-column; and

(b) adding the sample of individual units at one end of the product-column and selecting for the catalyst molecules of interest by isolating the individual unit(s) which arrive(s) latest to the opposite end on the column.

19. A method for producing a catalyst molecule of interest comprising performing the method for *in vitro* selection according to any of claims 1-18 and the further following step,

(a) producing said isolated catalyst molecule of interest in a suitable quantity of interest by a suitable production method.

Figure 1

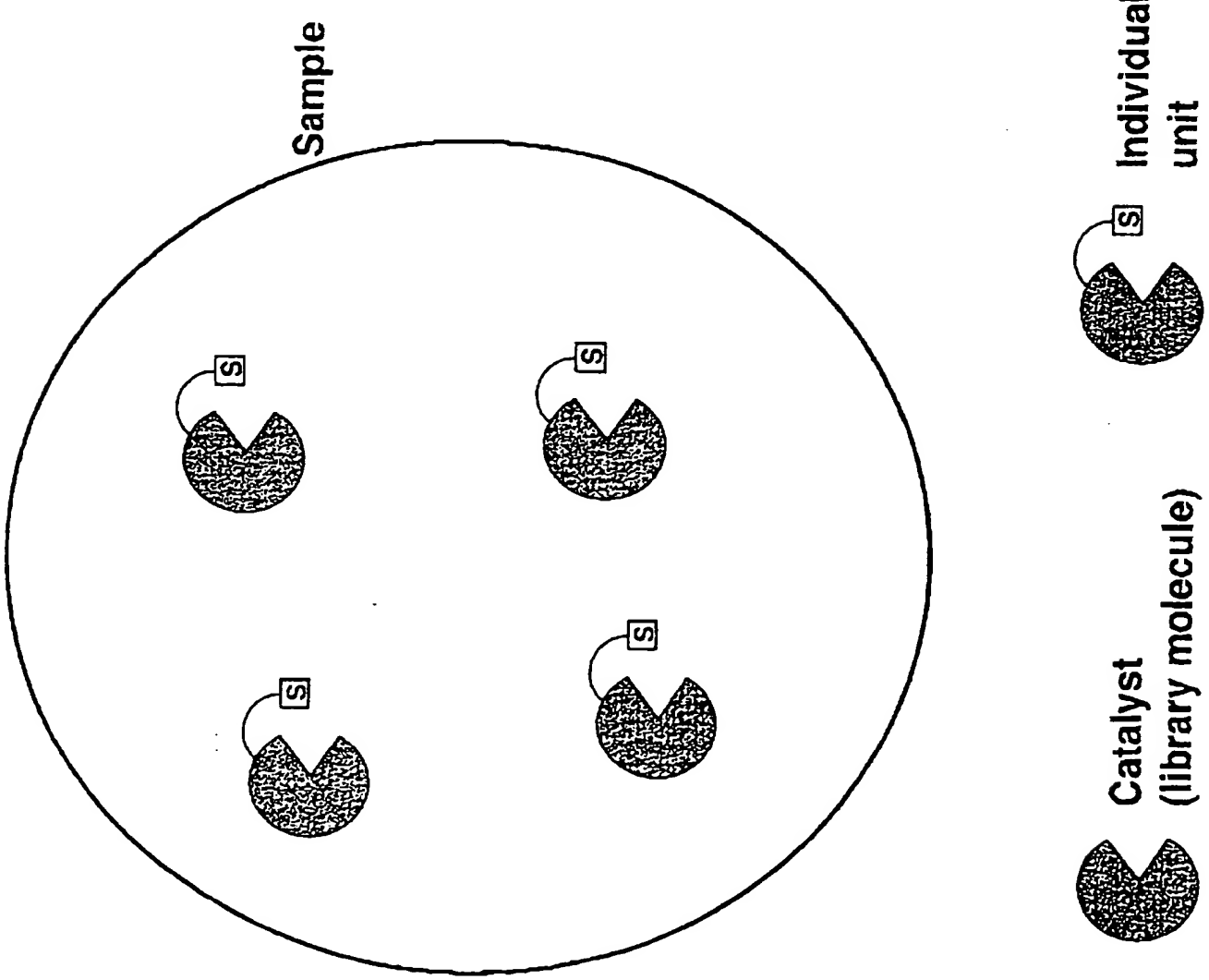


Figure 2

Figure 3

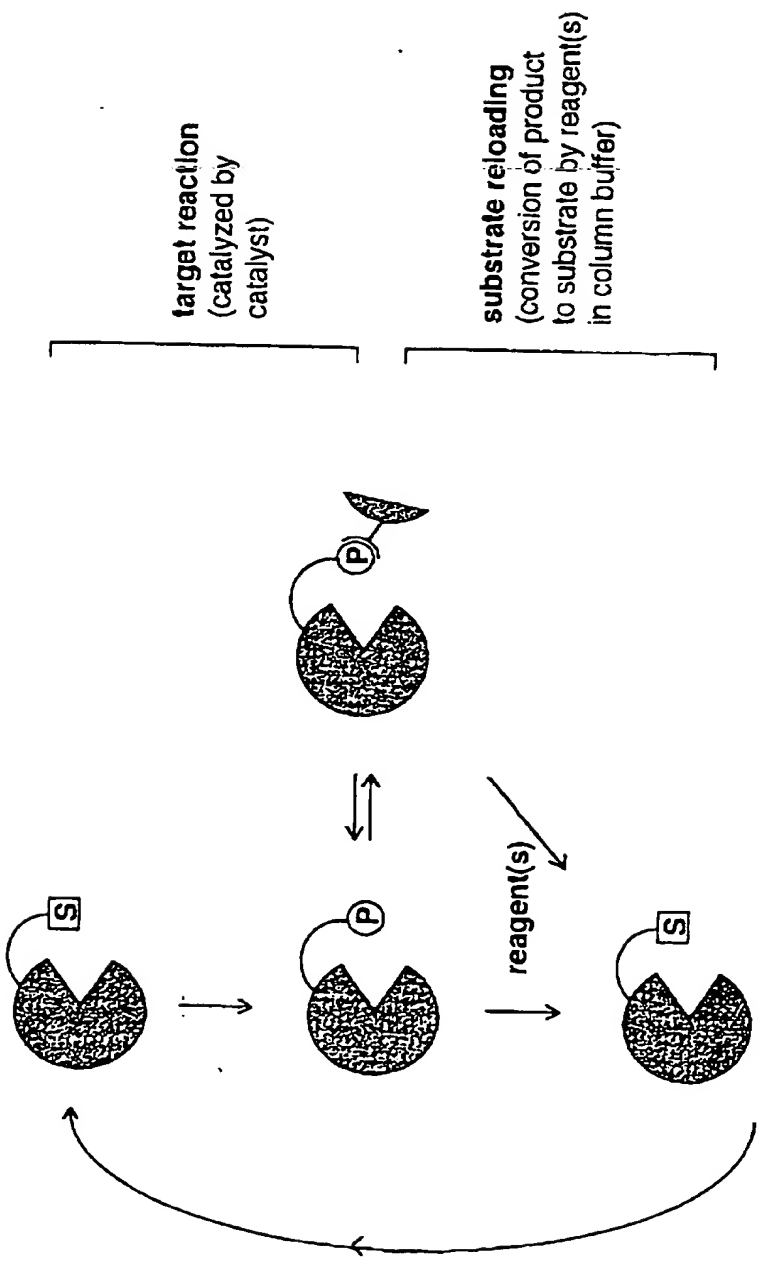

target reaction
(catalyzed by
catalyst)
substrate reloading
(conversion of product
to substrate by reagent(s)
in column buffer)
P
S
P
reagent(s)
S

Figure 4

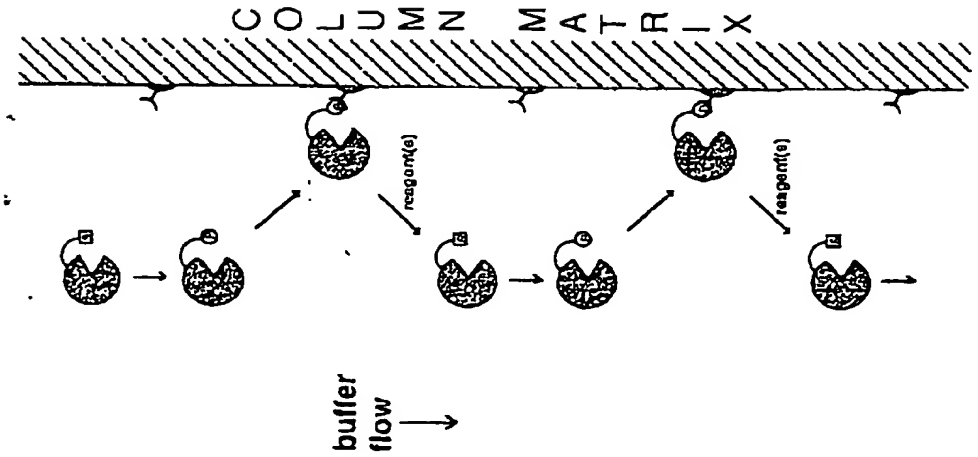


Figure 5

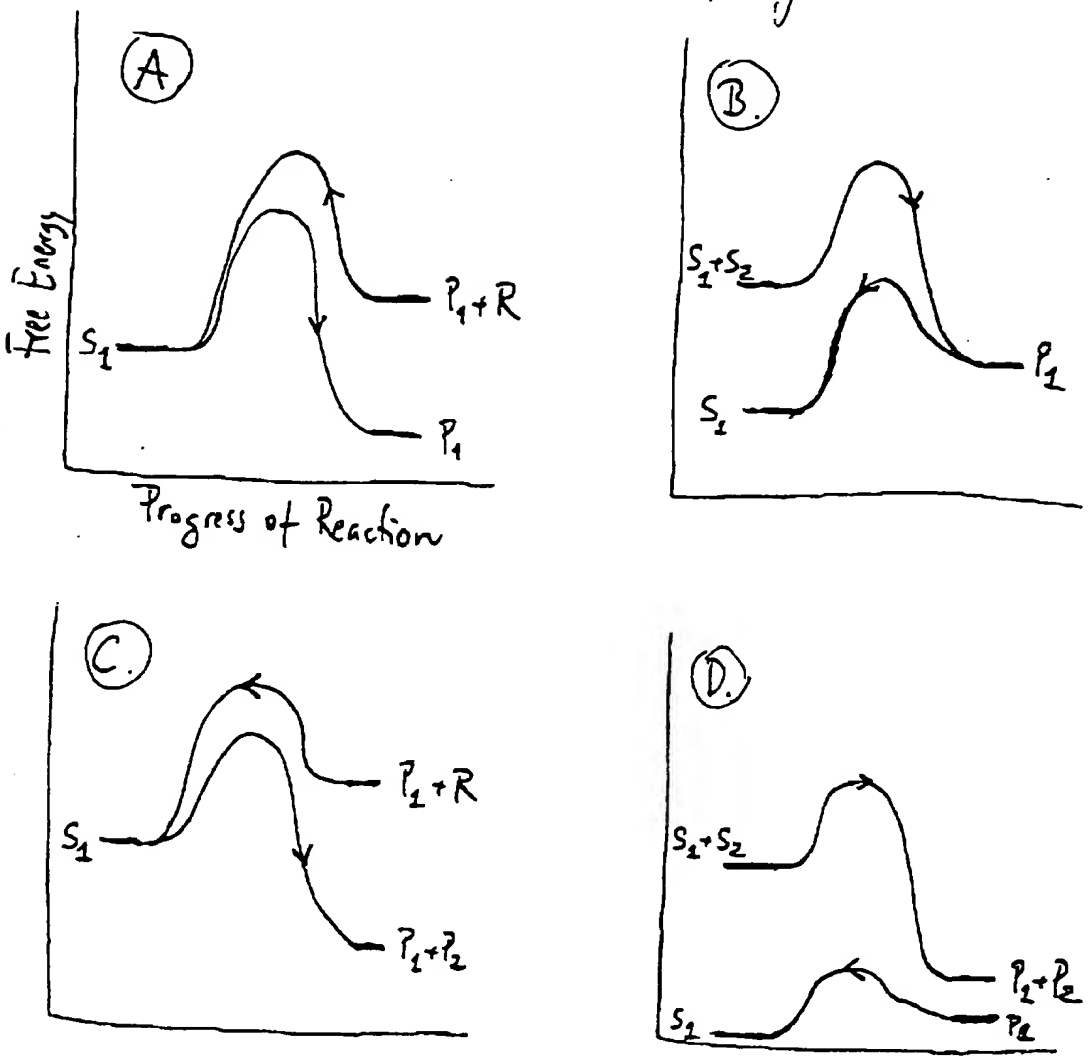


Figure definitions:
S_1: Substrate attached to individual unit prior to target reaction.
P1: Product attached to individual unit after target reaction has occured.
S2: All additional substrates of forward (target) reaction.
P2: Represents all additional products of forward reaction.
R: Reactants of the reverse reaction (reactants are thus reagents).

Catalysts (eg., enzymes) used as reagent(s) in the direct reloading protocol are as all other catalysts: They only lower the activation energy of the reaction. Consequently, the availability of an enzyme has no influence on whether a combination of target reaction and reagent(s) is feasible or not, but it does have an effect on selection stringency in the assay, because the enzyme will have an effect on the efficiency (speed) of the substrate reloading process.

Figure 11

Figure 11a

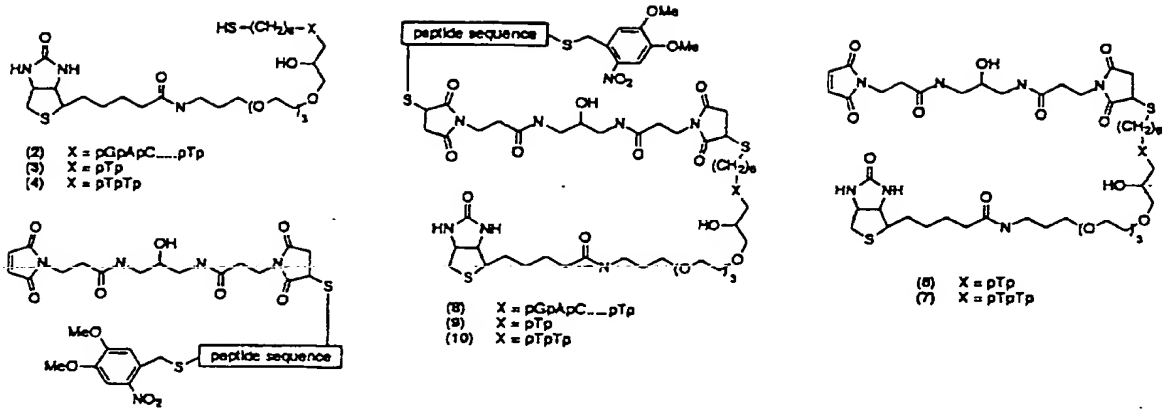


Figure 11b

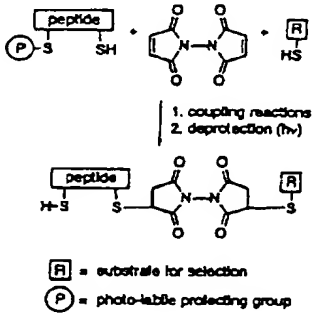


Figure 12

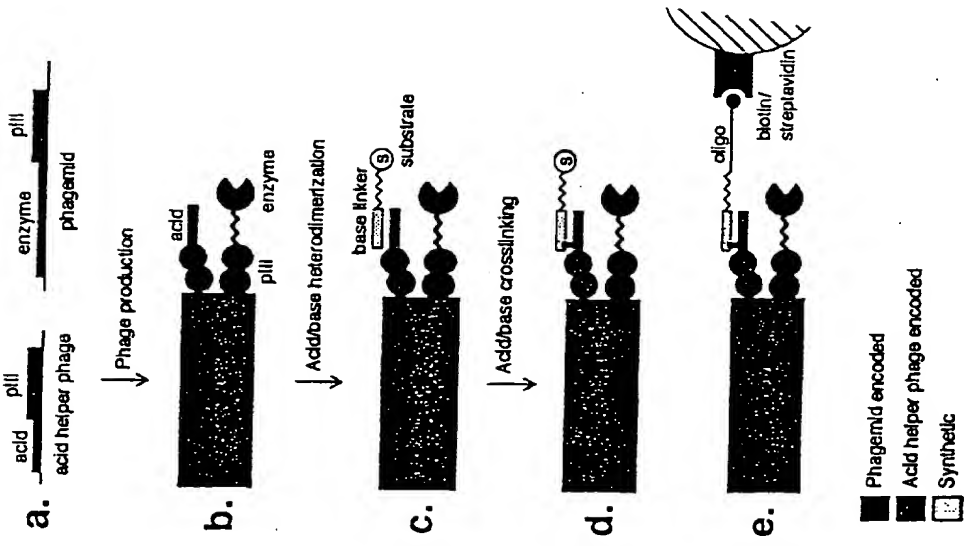
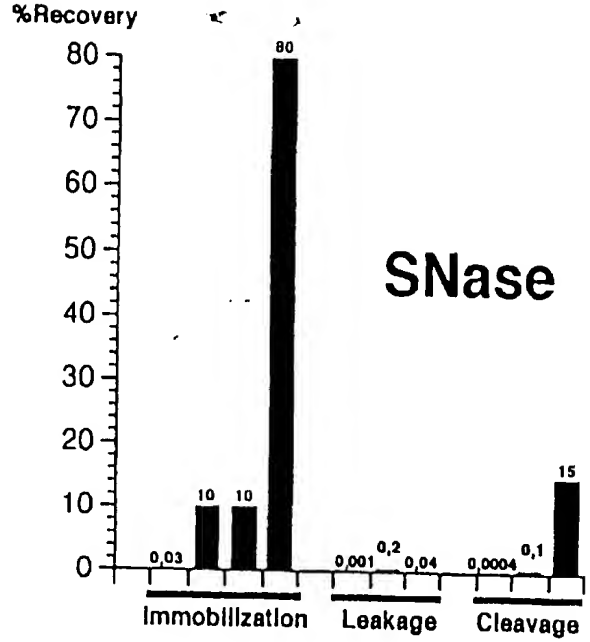


Figure 2

a.


%Recovery
80
70
60
50
40
30
20
10
0
80
10
10
0,03
0,001
0,2
0,04
0,0004
0,1
15
Immobilization
Leakage
Cleavage
SNase

b.

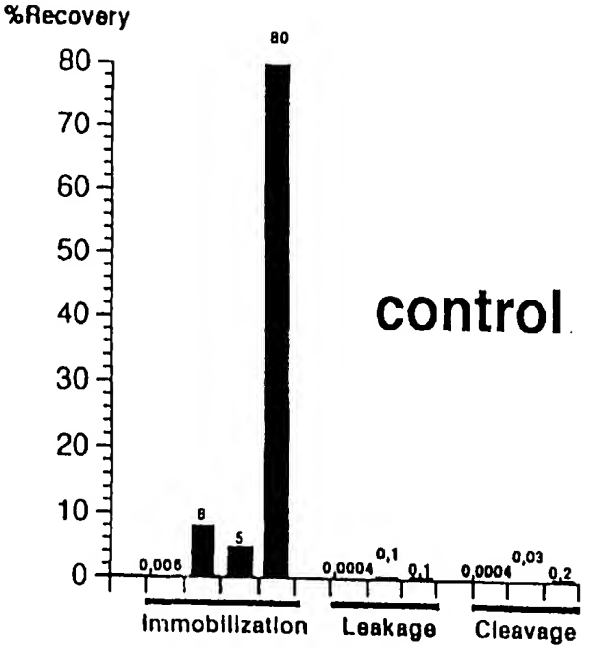

%Recovery
80
70
60
50
40
30
20
10
0
80
8
5
0,006
0,0004
0,1
0,1
0,0004
0,03
0,2
Immobilization
Leakage
Cleavage
control

Figure 13

Direct reloading.

Figure 14

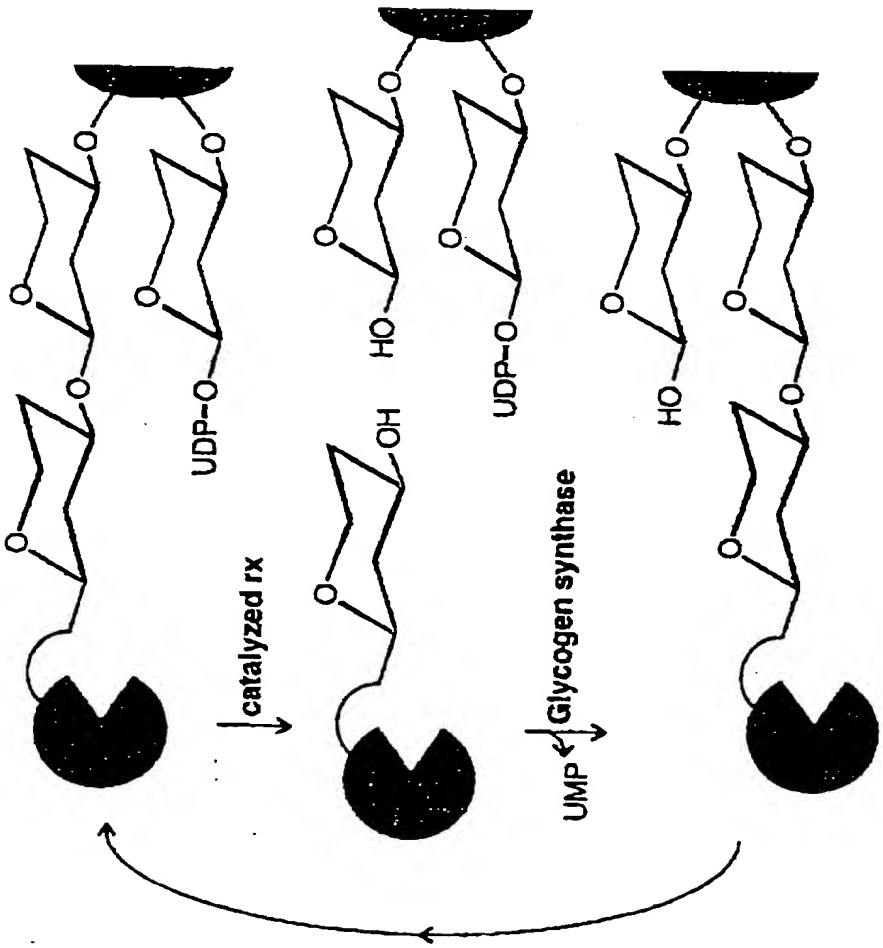


Fig. 7